(19) (CA)  **CANADIAN PATENT** (12)

(54) Human Anti-Rh(D) Monoclonal Antibodies

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ABSTRACT

Human Anti-Rh(D) Monoclonal Antibodies

The present invention provides human monoclonal
antibodies having the following essential
5   characteristics:

(a)  exhibiting activity against Rh(D) antigen, but not
     C, c, E or e antigens of the Rh blood group system;

(b)  being IgG1 proteins;

(c)  having kappa light chains;

10   (d)  being G1m (3) or G1m (1, 17) allotype;

(e)  exhibiting activity against $D^u$ cells by an indirect
     antiglobulin test;

(f)  exhibiting activity against $D^{IV}$, $D^V$ and $D^{VII}$
     variant antigens; and

15   (g)  being inactive against $D^{VI}$ or $D^B$ variant antigens,
which may be employed for Rh-typing of red blood cells
and passive immunisation to prevent haemolytic disease
of the newborn.

Cell lines capable of producing such monoclonal
20   antibodies were deposited at the European Collection of
Animal Cell Cultures, Porton Down, U.K. on 16th
September 1987 under accession numbers ECACC 87091605
and ECACC 87091604.

CLAIMS:

1. A human monoclonal antibody having the following essential characteristics:

5   (a)   exhibiting activity against Rh(D) antigen, but not C, c, E or e antigens of the Rh blood group system;

  (b)   being IgG1 proteins;

  (c)   having kappa light chains;

  (d)   being G1m (3) or G1m (1, 17) allotype;

10   (e)   exhibiting activity against $D^u$ cells by an indirect antiglobulin test;

  (f)   exhibiting activity against $D^{IV}$, $D^V$ and $D^{VII}$ variant antigens; and

  (g)   being inactive against $D^{VI}$ or $D^B$ variant antigens

15       and antigen-binding fragments thereof.

2. A monoclonal antibody as claimed in claim 1 selected from the monoclonal antibodies of the cell lines ECACC 87091605 and ECACC 87091604.

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3. An anti-Rh(D) reagent wherein a monoclonal antibody as claimed in claim 1 is combined with one or more further anti-Rh(D) antibodies having one or more additional binding specificities.

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4. An anti-Rh(D) reagent as claimed in claim 3 wherein a monoclonal antibody capable of binding the $D^{VI}$ variant is present.

30   5. An anti-Rh(D) reagent as claimed in claim 3 wherein the antibody component comprises:

  (a)   a monoclonal antibody as defined in claim 1, and

  (b)   an IgM anti-Rh(D) with no or only weak anti-$D^u$ activity. /

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6.    An anti-Rh(D) reagent as claimed in claim 5, wherein the antibody component additionally comprises an IgG monoclonal anti-Rh(D) capable of exhibiting activity against $D^{VI}$ cells in an indirect antiglobulin test.

7.    An anti-Rh(D) reagent as claimed in claim 5 wherein component (b) is selected from the monoclonal IgMs of the deposited hybridoma cell lines MAD-2 (ECACC 86041803) and FOM-1 (ECACC 87021301).

8.    A human lymphocyte-derived cell line capable of producing a monoclonal antibody as claimed in claim 1.

9.    A cell line as claimed in claim 8 selected from ECACC 87091605 and ECACC 87091604.

10.    A monoclonal antibody as claimed in claim 1 for use in passive immunisation to prevent haemolytic disease of the newborn.

11.    A monoclonal antibody as claimed in claim 10 of the allotype G1m(3).

12.    A culture supernatant obtained by cultivation of a cell line as claimed in claim 8 or claim 9.

13.    A pharmaceutical composition for use in passive immunisation to prevent haemolytic disease of the newborn comprising a monoclonal antibody as claimed in claim 1 in association with a physiologically acceptable carrier or diluent.

14.    A pharmaceutical composition as claimed in claim 13 wherein said monoclonal antibody is of the allotype G1m(3).

15. A pharmaceutical composition as claimed in claim 13 wherein the antibody component additionally comprises a monoclonal antibody capable of binding the $D^{VI}$ variant.

5  16. A pharmaceutical composition as claimed in claim 13 wherein the antibody component includes one or more anti-Rh(D) antibodies of the IgG3 sub-class.

17. A method of Rh-typing wherein a monoclonal antibody
10  as claimed in claim 1 or an anti-Rh(D) reagent as claimed in claim 3 is employed, said monoclonal antibody or said reagent being in the form of an aqueous solution.

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PK 51-900 (Kumpel 1)


Human Anti-Rh(D) Monoclonal Antibodies

The present invention relates to human monoclonal antibodies to the Rh(D) antigen of human red blood cells. In particular, it relates to such antibodies of the IgG1 sub-class which may be used to detect not only the normal Rh(D) antigen on either D-positive
5 or "weak D" or $D^u$ cells, but also important variants of the Rh(D) antigen.

Of the antigens of the so-called Rh blood group system, the Rh(D) antigen is responsible for some of the most severe reactions following
10 transfusion to a patient with corresponding antibody. Since an Rh(D-) individual with anti-Rh(D) who receives Rh(D+) blood is liable to suffer substantial red blood cell (RBC) destruction due to the Rh(D) phenotype incompatibility, blood of donors and
15 blood transfusion recipients is routinely classified as Rh(D+) or Rh(D-) by agglutination tests with anti-Rh(D) antibody. The Rh phenotype of RBCs is commonly further defined with reference to the Fisher-Race system, which is based on the assumption
20 that the inheritance of the Rh antigens is determined by three pairs of allelic genes, C-c, D-d and E-e, acting at very closely linked loci. According to this theory, a person may inherit a set of three Rh genes from each of his parents (i) C or c, (ii)
25 D or d, (iii) E or e (no d antigen has as yet been identified, but the symbol 'd' is used to indicate the presence of a gene, allelic to the D gene, which does not produce D antigen). For example, an Rh(D+) person may inherit CDe from one parent
30 and cde from the other. The frequencies of the commonest Rh gene combinations as determined with

reference to the Fisher-Race system for an English
population, together with the 'short symbols' which
are used, particularly in speech, are given in
Table 1 below.

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### Table I
### Frequency of common Rh genes for an English Population

| Short symbol | CDE nomenclature | Frequency (%) |
|---|---|---|
| $R^1$ | CDe | 40.8 |
| r | cde | 38.9 |
| $R^2$ | cDE | 14.1 |
| $R^0$ | cDe | 2.6 |
| $R^{1w}$ | $C^wDe$ | 1.3 |
| r" | cdE | 1.2 |
| r' | Cde | 0.01 |
| $R^z$ | CDE | rarer |
| $r^y$ | CdE | |
| $R^N$ | (C)D(e) | |

Despite expansion over the years, the Fisher-
Race system has not been adequate to account for
all the reactions that have been observed with
25 the Rh system (Mollison, P.L. (1983) Blood Transfusion
In Clinical Medicine, 7th edn., Blackwell Scientific,
Oxford). Nevertheless, the World Health Organisation
has recommended that in the interest of simplicity
and uniformity this nomenclature should be universally
30 adopted and all Rh genotypes given hereinafter
are defined on the basis of the conventional Fisher-
Race system.

In addition to the need for anti-Rh(D) antibody
for Rh-typing of RBCs, such antibody is also importantly
35 required for passive immunisation of Rh(D-) mothers
to prevent haemolytic disease of the newborn (HDN).
This condition arises in newborn Rh(D+) infants
of Rh(D-) mothers previously sensitized to Rh(D)

antigen as a result of IgG anti-Rh(D) antibodies
crossing the placenta during pregancy and causing
foetal RBC destruction.  Sensitization of the Rh(D-)
mother to Rh(D) antigen may have occurred at the

5    birth of an earlier Rh(D+) child due to some foetal
RBCs entering the maternal circulation and being
recognised by the maternal immune system.  To reduce
the incidence of HDN, it is routine practice in
the United Kingdom and many other countries to

10   give anti-Rh(D) antibodies to Rh(D-) mothers immediately
after the birth of an Rh(D+) infant so that any
Rh(D+) RBCs which have entered the maternal circulation
are rapidly removed (Mollison, P.L. (1983) loc.
cit.; Laros Jr., R.K. (1986), "Erythroblastosis

15   Fetalis" in "Blood Group Disorders In Pregnancy",
Ch. 7, p. 103).

At the present time, anti-Rh(D) antibody
for use in both Rh-typing of RBCs and passive immunisation
of Rh(D-) mothers is largely obtained directly

20   from female donors immunised during pregnancy or
from immunised male volunteers.  The success of
the programme of post-partum prophylactic administration
of human anti-Rh(D) immunoglobulin to Rh(D-) women
has, however, resulted in a dramatic reduction

25   in the number of naturally alloimmunised women
(Urbaniak, S.J., "RhD haemolytic disease of the
newborn: the changing scene", Br. Med. J. (1985)
291, 4-6).  Also, deliberate immunisation of individuals
with Rh(D+) RBCs carries the risks common to receiving

30   any transfusion of RBCs,e.g. risk of transmission
of hepatitis viruses and HIV.  Hence, there is
much interest in obtaining human monoclonal anti-
Rh(D) antibodies for both diagnostic and therapeutic
purposes.

35       As stated above, in routine blood testing,
blood types are divided into Rh(D+) and Rh(D-)
on the basis of the apparent presence or absence

of Rh(D) antigen on the RBCs as indicated by agglutination
tests with anti-Rh(D). However, a small number
of persons with apparently Rh(D-) blood have RBCs
that are not directly agglutinated by anti-Rh(D)
5 during such routine testing, but that do react
when the D-typing test is performed using selected
anti-Rh(D) reagents by the indirect antiglobulin
test. Cells thus identified are designated $D^u$.
The frequency of the $D^u$ phenotype is about 0.2%
10 overall, 0.6% among Caucasians, and about 1.5%
of all Rh(D-) gravid women. At least three different
mechanisms may be responsible for the expression
of the $D^u$ phenotype: (1) hereditary absence of
a portion of the complete Rh(D) antigen, (2) gene
15 interaction with suppression of $\underline{D}$ by $\underline{C}$ in the trans
position, and (3) a D gene producing a weak antigen.

In the early 1950s, reports first appeared
of the presence of anti-Rh(D) in individuals of
the $D^u$ phenotype following blood transfusion with
20 Rh(D+) blood or pregnancy resulting in the birth
of a Rh(D+) infant. It later became apparent that
in some individuals whose blood is classified Rh(D+)
parts of the Rh(D) antigen are missing from the
RBCs. When exposed by transfusion or pregnancy
25 to Rh(D+) RBCs carrying the complete Rh(D) antigen,
persons carrying an incomplete Rh(D) antigen on
their RBCs are capable of making alloanti-D against
the Rh(D) antigen portion they lack. The blood
of such individuals is called D variant when the
30 RBCs react directly with routine anti-Rh(D) reagents
or $D^u$ variant when the cells react only by the
indirect antiglobulin technique.

The observation that allo anti-Rh(D) can
be produced in patients who have Rh(D+) RBCs has
35 led to common usage of the term "D mosaic" to describe
the Rh(D) antigen in its complete native form.
Routine anti-Rh(D) reagents generally cannot differentiate

those RBCs that lack part of the D mosaic from those
that have all the D components.

The D variant phenotypes have been categorised by
Tippett and Sanger (Vox. Sang. (1962)$\underline{7}$, 9-13). This
system is based on the interaction of RBCs and serum
from D- and $D^u$ variant individuals. The seven
categories (see Table II below) allow for expansion;
subdivisions are already recognised in categories III<
IV and V. Categories I and II have been found to have
so many similarities that they are now generally
considered as a single sub-group.

### Table II
### Tippett and Sanger categories for D- or
### $D^u$positive blood with anti-Rh(D)

| Category | Racial origin | Usual haplotype |
|---|---|---|
| I | White | $\underline{DCe}$ |
| II | | |
| IIIa | Black | |
| IIIb | Usually Black | $\underline{Dce}$ |
| IIIc | White | |
| IVa | Mostly Black, some White | |
| IVb | White | $\underline{Dce}$ |
| Va | | Black and White |
| Vb | White | $\underline{D^uCe}$ |
| Vc | Black and White | |
| VI | Nearly all White | $\underline{D^uCe}$ |
| VII | White | $\underline{DCe}$ |

An alternative, but lesser used, classification by
Wiener uses letters A,B,C,D instead of Roman Numerals.
Although there is no direct correlation between the two
systems, it is often considered that $D^B$ and $D^{V1}$ are
interchangeable.

Although the frequency of D and $D^u$ variant
individuals within the human population is relatively

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low, the total number of individuals of these blood
types who potentially have some risk of effective
anti-Rh(D) formation as a result of exposure by blood
transfusion or pregnancy to non-variant Rh(D+) cells is

5   far from insignificant. Moreover, in addition to Rh(D-)
women who give birth to Rh(D+) or $D^u$ infants, $D^u$ variant
women who give birth to an Rh(D+) infant may also
benefit from post-partum anti-Rh(D) treatment to reduce
the risk of HDN (White, C.A. et al. (1983) Am. J.

10  Obstet. Gynecol. 145, 1069-1073). Anti-sera capable of
distinguishing D and $D^u$ variant RBCs are not widely
available. Hence, provision of anti-Rh(D) monoclonal
antibodies with a range of binding specificities for D
and $D^u$ variant RBCs is seen as useful in enabling the

15  more ready identification and categorisation of
individuals possessing such cells (especially D or $D^u$
variant pregnant females who are suitable candidates for
prophylactic anti-Rh(D) treatment) as well as for
providing further structural information on the Rh(D)

20  antigen complex.
     Human monoclonal anti-Rh(D) antibody production has
previously been achieved by:-
(a)   directly cloning Epstein Barr virus transformed B
lymphocyte cell lines (hereinafter referred to as

25  EBV-transformed LCL) derived from B lymphocytes of
anti-Rh(D) positive donors (see GB-A 2127434 of
Crawford, published 11 April 1984; Crawford et al.
(1983) Lancet 1, 386-388 and Paire et al (1986) Immunol.
Lett. 13, 137-141),

30  (b)   cloning hybridoma cell lines formed by fusing
anti-Rh(D) producing, EBV-transformed LCL with mouse,
mouse-human or human myeloma cell lines (see co-pending
British application of Central Blood Laboratories
Authority, published under no. GB-A-2189506 on 28

35  October 1987, Thompson et al. (1986) Immunol. 58,
157-160 and International Patent Application publication
No. WO85/02413 of The Board of Trustees of the Leland

Stanford Jr. University, published on 6 June 1985), or
(c) by fusion of a human LCL with immune B cells (Lowe
et al (1986) Vox. Sang. 51, 212-216).

By cloning EBV-transformed LCL from 3 anti-Rh(D)

5   positive donors, we have been able to obtain, however,
monoclonal anti-Rh(D) antibodies of the IgG class which
have a particularly useful binding specificity spectrum
not shown for any previously disclosed anti-Rh(D)
monoclonal antibody reagent.

10   According to the present invention, we provide
human monoclonal antibodies having the following
essential characteristics:
(a)  exhibiting activity against Rh(D) antigen, but not
C, c, E or e antigens of the Rh blood group system;

15  (b)  being IgG1 proteins;
(c)  having kappa light chains;
(d)  being G1m (3) or G1m (1, 17) allotype;
(e)  exhibiting activity against $D^u$ cells by an indirect
antiglobulin test;

20  (f)  exhibiting activity against $D^{IV}$, $D^V$ and $D^{VII}$
variant antigens; and
(g)  being inactive against $D^{VI}$ or $D^B$ variant antigens,
and antigen-binding fragments thereof.

Such monoclonal antibodies can be employed as

25  routine anti-Rh(D) reagents to classify RBCs as Rh(D+),
$D^u$ or Rh(D-).

For this purpose, a monoclonal antibody of the
present invention may be employed either alone or in
combination with one or more further anti- Rh(D)

30  antibodies, preferably monoclonal antibodies, having one
or more additional binding specificities.  Thus, for
example, a monoclonal antibody of the present  invention
may be advantageously blended with a further  monoclonal
antibody capable of binding the $D^{VI}$ variant  to provide

35  an anti-Rh(D) reagent of broader specificity capable of
classifying $D^{VI}$ variant RBCs as D-positive. In such an
anti-Rh(D) reagent, an IgG1 antibody of the present

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invention may, for example, be combined with an IgG1
monoclonal antibody of the type disclosed in our
International patent application published under No.
WO89/02443 on 23 March 1989 having the following binding
5    characteristics:

     (a)    exhibiting activity against Rh(D) antigen,
          but not C, c, E or e antigens of the Rh blood
          group system;

     (b)    exhibiting activity against $D^V$, $D^{VII}$ $D^{VI}$ and
10          $D^B$ variant antigens; and

     (c)    being substantially non-reactive with
          non-papain treated $D^{IV}$ cells in an IAG test.

    Amongst such antibodies, particularly preferred for
use in combination with an anti-Rh(D) monoclonal
15    antibody of the present invention is the monoclonal
antibody designated B7 deposited at the European
Collection of Animal Cell Cultures, Porton Down, U.K.
under accession No. ECACC 87091603 on 16th September
1987.

20    If monoclonal antibodies of the present invention
are used for Rh-typing in parallel with an anti- Rh(D)
reactive against the $D^{VI}$ variant, eg. an appropriate
polyclonal anti-Rh(D) serum, those blood samples giving
a positive result in an agglutination test with the
25    latter, but negative results with a monoclonal antibody
of the invention can be predicted to be mainly or
entirely of the $D^{VI}$ category (since this is virtually
the only D variant antigen against which the new
monoclonal antibodies are inactive). It has been
30    established that amongst individuals classified as
Rh(D+) or $D^u$ by a conventional agglutination test, but
who are capable of making anti-Rh(D), a high percentage
have the $D^{VI}$ variant antigen (Mollison, P.L. (1983) in
"Blood Transfusion In Clinical Medicine", Ch. 8, p 339).
35    One use of the monoclonal antibodies of the invention
is, indeed, in investigating the incidence of
individuals of the $D^{VI}$ type in the population.

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A monoclonal antibody of the present invention may also be of particular value for use in an anti- Rh(D) typing reagent to supplement the specificity of an anti-Rh(D) with no or only weak anti-$D^u$ activity, i.e.

5    insufficient activity against $D^u$ cells to be able to reliably distinguish such cells from D-negative cells in a conventional agglutination test. Indeed, under FDA regulations in the U.S.A. governing commercial anti-Rh(D) typing reagents, it is obligatory for such a

10   reagent to be able to distinguish $D^u$ RBCs from truly D-negative RBCs. Especially preferred amongst combination anti-Rh(D) reagents of the present invention are such reagents satisfying the above condition wherein an IgG anti- Rh(D) of the invention is employed together

15   with an IgM anti-Rh(D) with no or only weak $D^u$ activity, e.g. an IgM monoclonal anti-Rh(D) selected from the monoclonal IgMs of the deposited hybridoma cell lines MAD-2 (ECACC 86041803) and FOM-1 (ECACC 87021301), which form inter alia, the subject matter of published

20   European Patent Application 0251440 of Central Blood Laboratories Authority, published on 7 January 1988. Such a reagent will advantageously be further complemented with at least one further IgG monoclonal anti-Rh(D) antibody which individually exhibits activity

25   with $D^u$ red cells by the indirect antiglobulin test, such that the blended reagent reacts by the same test with $D^u$, $D^{IV}$, $D^V$ and $D^{VI}$ cells. When Rh typing is carried out with such a reagent, D-positive cells will firtly be directly agglutinated by the IgM anti-Rh(D).

30   The remaining non-agglutinated cells (apparently D-negative) may then be subsequently divided into truly D-negative and $D^u$ cells by addition of conventional Coomb's reagent for an indirect antiglobulin test, whereupon $D^u$ cells binding IgG antibody will be

35   agglutinated and thus distinguished.

The monoclonal anti-Rh(D) antibodies of the invention can be made by conventional methods known for

the production of monoclonal antibodies and in particular by the culture of EBV-transformed human B-lymphocytes selected on the basis of secretion of anti-Rh(D) immunoglobulin having the characteristics set

5 out above for the required antibodies. The culture supernatants so produced constitute a further feature of the present invention.

We have now investigated in detail 9 cloned EBV-transformed LCL which produce IgG1 anti-Rh(D)

10 monoclonal antibodies as defined above. All these cloned cell lines were obtained by starting with peripheral B lymphocytes from 2 chosen anti-Rh(D) donors and employing the procedure described in GB-A 2145113 of UK Secretary of State for Social Services, published on

15 20 March 1985 or a substantially similar procedure to establish and clone EBV-transformed LCL producing a monoclonal antibody of the desired specificity (see Example 1). In continuous culture using RPMI- 1640 medium supplemented with 10% (v/v) mycoplasma free-fetal

20 calf serum, 0.2 mg/ml arginine and antibiotics to prevent mycoplasma growth, they have been found to be highly stable and to provide a culture supernatant having an anti-Rh(D) titre, as determined by an indirect antiglobulin (IAG) assay in low ionic strength saline

25 versus $R_1R_1$ (CDe/CDe) RBCs, in the range 2000-8000. Such a culture supernatant is suitable for use in Rh-typing without the need for concentration and indeed may be diluted for use. Four of the selected cell lines (A1, A2 and A3 derived from a donor A) have been shown

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to maintain a stable anti-Rh(D) titre in continuous
culture for over 2 years. Four further cell lines
capable of providing a culture supernatant as above
(B1, B2, B3 and B11 derived from a donor B) have
5  been maintained in continuous culture for over
8 months without substantial decrease of anti-Rh(D)
titre. Antibody production characteristics of
the above-mentioned specific clones in continuous
culture are summarised in Tables IIIa and IIIb
10  below.

## Table IIIa

| IAG titre (3% RBCs in low ionic strength saline) | |
|---|---|
| 15  $R_1r$ cells | 64-512 |
| $R_1R_1$ cells | 2000-8000 |
| $R_1^u r$ cells | 8-512 |
| 20 | |
| Microtitre with bromelain-treated $R_1R_2$ cells | 33,000-131,000 |
| 25  Anti-Rh(D) (IU/ml) | 10-98 |
| IgG (µg/ml) | 3.9-11.0 |

Table IIIb

| culture | A1 | A2 | A3 | B1 | B2 | B3 | B11 | B12 | B13 |
|---|---|---|---|---|---|---|---|---|---|
| Supernatant | IAG reactivity of supernatant with 3% RBCs in low ionic strength saline. (Grade: 0 to 6) | | | | | | | | |

| RBC phenotype | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| $R_1R_1$ | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 |
| $R_1{}^u r$ | 6 | 6 | 6 | 6 | 6 | 4 | 5 | 6 | 6 |
| $R_o{}^u r$ | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 |

It was further found that those monoclonal
antibodies of the invention that were tested reacted
by IAG with RBCs of phenotypes $R_2 rG-$, $hr^S-$, $R_1R_z$
and $R_2R_z$ but are negative with $r''Gr$, $r^m$, $r^G$, $r'^S r$,
$hr^B-$, $r'^w r$ and $Rh33+$.

5      Gm allotyping of the antibodies of clones
A1, A2, A3, B1, B2, B3, B11, B12 and B13  has shown
that they fall into two allotype groups.  Using
the W.H.O.  (1974) notation, the IgG1 anti-Rh(D)
antibodies of clones A1, A2 and A3 were found to

10 be of the Glm (1, 17) allotype, whereas the antibodies
of the remaining above-mentioned clones derived
from donor B were found to be of the allotype Glm(3).
The latter antibodies are of particular interest
from the point of view of providing a monoclonal

15 antibody preparation for use in post-partum immunisation
of Rh(D-) mothers.  In general, the antibodies
in the Glm (1, 17) allotype group were active against
the $\bar{\bar{R}}^N$ antigen, whereas those of Glm(3) allotype
were not.

20      IgG1 anti-RhD antibodies are generally poor
promoters of phagocytosis of Rh(D+) RBCs by monocytes

and macrophages. However, we have found that IgG1
anti-Rh(D) antibodies of the Glm(3) allotype, e.g. the
IgG1 antibodies of clones B1, B2, B3, B11, B12 and B13,
unlike IgG1 anti-Rh(D) antibodies of the Glm(1,17)
allotype, are highly effective in mediating lysis of
sensitised RBCs by K lymphocytes in an antibody-directed
cell cytoxicity (ADCC) assay. This is in keeping with
the previously reported observation of Parinaud et al.
that foetal haemolysis is more severe with maternal IgG1
antibodies of the Glm(4) allotype, i.e. Glm(3) allotype
according to the W.H.O (1974) nomenclature (Am. J.
Obstet. Gynecol (1985) 1111-1115).

Thus, according to a further aspect of the present
invention, we provide a monoclonal IgG1 antibody of the
present invention, preferably a monoclonal antibody of
the present invention having the allotype Glm(3), for
use in passive immunisation of an Rh(D-) or D- or $D^u$-
variant mother after the birth of an Rh(D+) child to
prevent sensitisation of the mother to Rh(D) antigen. A
sterile solution of such an antibody for human injection
may be formulated in any physiologically acceptable
aqueous medium, for example isotonic phosphate buffered
saline or serum. Alternatively, the antibody may be
supplied in a freeze-dried formulation ready for
reconstitution prior to use. To provide a highly
efficient prophylactic preparation for use in the
prevention of HDN, a monoclonal anti-Rh(D) of the
present invention, especially such an antibody of the
Glm(3) allotype, may desirably be employed with one or
more further anti-Rh(D) antibodies, for example one or
more further anti-Rh(D) antibodies promoting
phagocytosis of Rh(D+) RBCs in vivo, e.g. an anti-Rh(D)
monoclonal antibody of the IgG3 sub-class, such as an
lgG3 monoclonal anti- Rh(D) of our co-pending Canadian
Application No. 577600 of

even date herewith. The aforementioned IgG3 monoclonal anti-Rh(D) antibodies are exemplified by the monoclonal antibody of the deposited cell line ECACC 87091606. For routine use, ideally a prophylactic pharmaceutical

5   composition of the present invention will include an anti-$D^{VI}$ antibody.

According to a still further aspect of the present invention, we provide a method of Rh-typing of RBCs wherein an aqueous solution of a monoclonal anti-Rh(D)

10   immunoglobulin of the present invention is employed. The monoclonal immunoglobulin is preferably contained in a culture supernatant which may be used directly or, more usually, after dilution. Particularly preferred for use in Rh-typing are culture supernatants containing

15   monoclonal anti- Rh(D) immunoglobulin according to the present invention which will agglutinate at high dilution (e.g. 1:1000 dilution) enzyme-treated RBCs carrying the Rh(D) antigen and in the IAG test will agglutinate $D^{u}$ RBCs at, for example, 1:10 dilution.

20   As hereinbefore indicated, it may be desirable to blend an IgG1 antibody of the present invention with one or more further anti-Rh(D) monoclonal antibodies of different specificity, e.g. a further IgG1 antibody having anti-$D^{VI}$ activity. Suitable diluents include

25   physiological saline or phosphate buffered saline, advantageously containing bovine serum albumin and a surfactant or suspending agent such as Tween 80 (Trade Mark) or methyl cellulose.

The cell lines A3 and B2 were deposited on 16th

30   September 1987 at the European Collection of Animal Cell Cultures, Porton Down, U.K. under accession numbers ECACC 87091605 and ECACC 87091604 respectively.

Further details of the preparation of the above-noted deposited cell lines of the present

invention and the identifying characteristics of
the culture supernatants obtainable by continuous
culture of these are provided in Example 1 of the
following non-limiting examples.

EXAMPLE 1

(i) Establishment and Cloning of Anti-Rh(D) Producing
EBV-transformed LCL

(a) Sources of B Lymphocytes

Donor A: female, immunised during her first
and only pregnancy (which resulted in delivery
of a normal Rh(D+) infant), boosted with 0.5 ml
packed Rh(D+) ($R_2r$) RBCs 4 years after parturition,
and a peripheral blood sample obtained 8 days after
boosting when her serum anti-D level was 60IU/ml.
Donor B: male, initially immunised by transfusion
in 1966, boosted 6 times since and last boosted
13 days before donating a "buffy coat" fraction
(white cells) in 1985 when his serum anti-D level
was 318IU/ml.

(b) Establishment of cell lines

Peripheral blood mononuclear cells from donor
B were separated on Lymphoprep (Nyegaard and Co),
incubated in the presence of EBV (1 ml culture
supernatant from filtered mycoplasma free B95-8
cell line per $10^7$ cells) at 37°C for 1 hour and
washed in phosphate-buffered saline (PBS). Aliquots
were either
(a)    plated at $0.5 \times 10^6$ cells/ml in 2 ml wells
using lymphoblastoid cell culture medium (RPMI-
1640 medium containing 10% (v/v) mycoplasma free
foetal calf serum (FCS), 0.2 mg/ml arginine, 100 IU/ml
penicillin (Glaxo), 50 µg/ml streptomycin (Glaxo),
25 IU/ml polymixin (Glaxo), 25 µg/ml kanamycin
(Gibco), 20 µl/ml fungizone (Squibb), 25 µg/ml
gentamycin sulphate (Sigma) and 20 µg/ml trobicin
(UpJohn)), supplemented with either 1% (v/v) phytohaemag-

glutinin (PHA) or 0.5 µg/ml cyclosporin A (CsA);
or

(b)   enriched for surface anti-D positive lymphocytes
by rosetting with bromelain-treated $OR_1R_2$ (CDe/cDE)
5  RBCs prior to plating out as above.

Four cell lines from donor B were set up:
LCL and enriched LCL with either initial PHA or
CsA supplementation.

Isolated peripheral blood mononuclear cells
10  from donor A were similarly infected with EBV.
After washing with PBS, the cells were resuspended
in lymphoblastoid cell culture medium supplemented
with 1% (v/v) PHA and dispensed into 2 ml wells
above a feeder layer of mouse peritoneal macrophages.
15  All the cultures were subsequently incubated
at 37°C in 5% $CO_2$, 95% humidified air.  Medium
changes were performed every 3 to 4 days and, after
3 weeks culture, the cells were transferred to
50 ml flasks.  All the cell lines (except that
20  giving rise to clone B3) were enriched by rosetting
at 3-4 weekly intervals.

(c)   Cloning

25    Cells were plated out at limiting dilution
at 5 and 10 cells per well in flat bottomed 96-
well plates over a feeder layer of mouse peritoneal
macrophages (Doyle et al. (1985) Human Immunology
13, 199-209).  Cultures were fed once a week and
30  after 3-4 weeks cloned cells positive for anti-
D were grown up.

(d)   Derivation of clones

35

Three clones producing anti-Rh(D) antibody
of the IgGl sub-class (designated Al, A2 and A3)

were derived from B lymphocytes of donor A. The
polyclonal cell line giving rise to these clones was
initiated with PHA and sequentially enriched for
anti-Rh(D) positive cell by rosetting 13 times before
5    cloning.

Five further clones producing anti-Rh(D) antibody
of the IgG1 sub-class (designated $B_1$, $B_2$, $B_3$, B11 and
B12) were derived from B lymphocytes of donor B via a
polyclonal cell line initiated with PHA. B1 and B2 were
10   derived from cell lines repeatedly (6x) selected by
rosetting after establishing an LCL. B11 and B12 were
derived from a cell line rosetted only twice and B3 was
produced from an LCL maintained for 1 year without
rosetting before cloning. [It should be noted that B11
15   was erroneously designated C1 in the priority
application for this case (No. GB-A 8722018) of Central
Blood Laboratories Authority, filed 18 September 1987].

A further anti-Rh(D) IgG1 producing clone (B13) was
derived via a donor B polyclonal cell line supplemented
20   with CsA. This cell line was established from
lymphocytes selected for anti- Rh(D) immediately after
transformation by EBV and was subsequently re-rosetted
four times.

The results of tissue type and karotype analysis of
25   the above selected cell lines are set out in Table IV
below.

Table IV

| Cell Line | A1 | A2 | A3 | B1 | B2 | B3 | B11 | B12 | B13 |
|-----------|----|----|----|----|----|----|-----|-----|-----|

| | | |
|---|---|---|
| Donor | A | B |
| Sex | Female | Male |
| Tissue Type | | |
| HLA: A | 2,w19 | 1,3 |
| B | 44,35 | 8,35 |
| DR | 3,5 | 1,3 |
| Karotype | | |
| sex chromosomes | xx | xy |
| ploidy | most cells diploid | most cells diploid |

(e)     Quantitation of anti-Rh(D) activity and
IgG in culture supernatants

Anti-Rh(D) activity in the supernatants was
quantified against British national standards by Auto
Analyser. The mean of at least two determinations was
calculated. The quantitative estimation of IgG was
performed by ELISA (modification of the method of
Wakefield et al. in Clin. Chim. Acta. (1982) 123,
303-310) with at least eight determinations for each
supernatant. Coating antibody (affinity purified goat
anti-human IgG (Sigma)) was used at 1/200 in 0.05M
carbonate buffer pH 9.6. Supernatants and standard
(purified human IgG (Sigma)) were diluted in RPMI 1640 +
10% FCS. Peroxidase-conjugated goat anti-human IgG
(Sigma) was diluted 1/500 in PBS + 0.05% Tween 20 (Trade
Mark) and the substrate was TMB (3,3', 5,5'-tetramethyl
benzidine).

Not all the cell lines established under different
experimental conditions showed stable antibody
production. However, all the cell lines

which were subsequently cloned had maintained high
titres (over 1/33,000 by microtitre) of anti-D for over
6 months.  All clones from these cell lines were
positive for anti-D and maintained their titres

5    throughout the duration of continuous culture (A1, A2
and A3 - over 2 years; B1, B2, B3 and B11 - over 8
months).  The doubling time was 3-7 days. The cells grew
well in suspension culture with no loss of antibody
production.

10

(f)      Immunoglobulin class and subclass
determination

An immunodot assay (McDougal et al. J. Imm. Meth,
15   (1983) 63, 281-290) was used to determine the reaction
of the monoclonal anti-Rh(D) antibodies absorbed to
nitrocellulose with anti-IgG, anti-IgM, anti- kappa and
anti-lambda antiserum (Serotec); positive reactions were
detected with peroxidase-conjugated anti-sheep IgG
20   (Serotec) followed by colour development with
4-chloro-1-naphthol.  The IgG subclass was evaluated by
agglutination of anti-D coated RBCs by monoclonal
anti-subclass antibodies (Unipath).
The presence of single discrete bands for antibody
25   derived from the selected cell lines after
SDS-polyacrylamide gel electrophoresis was evidence of
monoclonality.

(g)      SDS-Page and Western Blotting.
30         Iscove's supernatants (serum free) were
electrophoresed under reducing conditions on 15%
polyacrylamide gels (Laemmli, Nature (1970) 227,
680-685).  The separated proteins were then
electrophoretically transferred to nitrocellulose
35   membranes (Burnette, Annals Biochem. (1981) 112,
195-203), which were probed with anti-IgG antiserum
(Serotec) and detected as above.

[A]

(h)      Protein A absorption

2 ml volumes of supernatants were run
twice down a 25 mm (1ml) column of Protein A Sepharose
Cl-4B (Trade Mark) (Sigma) and absorption of anti-Rh(D)
5    assessed by titration.

(i)      Gm allotyping

RBCs were coated with the monoclonal anti- D
10   antibodies and agglutination assessed using panels of Gm
allotyping reagents (Birmingham or Amsterdam).
The Gm allotypes of the monoclonal antibodies of
clones A1, A2, A3, B1, B2, B3, B11, B12 and B13 together
with other characteristics are given in Table V below.
15

Table V

Identification of immunoglobulin class,
subclass, and Gm allotype

| Monoclonal antibody: | A1 | A2 | A3 | B1 | B2 | B3 | B11 | B12 | B13 |
|---|---|---|---|---|---|---|---|---|---|
| Anti-IgG: | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ |
| anti-IgM: | − | − | − | − | − | − | − | − | − |
| anti-kappa: | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ |
| anti-lambda: | − | − | − | − | − | − | − | − | − |
| Anti-IgG1: | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 |
| anti-IgG2: | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| anti-IgG3: | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| anti-IgG4: | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Heavy chain $M_r$ (kDa): | 55.5 | 55 | 55 | 54 | 54 | 54 | 55 | * | * |
| Protein A absorption | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ |
| Gm allotype: | G1 m(1,17) Km-1 | G1 m(1,17) Km-1 | G1 m(1,17) Km-1 | G1 m(3) Km-1 | G1 m(3) Km-1 | G1 m(3) Km-1 | G1 m(3) Km-1 | G1 m(3) Km-1 | G1 m(3) Km-1 |

* not determined

(ii)  Serology

Anti-D titration was performed by adding
50 μl of 0.1% suspension of bromelain-treated $OR_1R_2$
(CDe/cDE) erythrocytes to 50 μl of supernatant,
5   neat and serially diluted two-fold, in V-well microplates.
After incubation at 37°C for 60 mins, and centrifugation
at 600 rpm for 3 mins, they were read macroscopically
by tilting the plate at 70° and allowing negative
reactions to trail.  The degree of agglutination
10  was graded in conventional manner and the titres
given as the highest dilution giving complete agglutination
(see Table IIIa).   Anti-D titres were also determined
by the IAG test using rabbit anti-human IgG and
3% $R_1r$, $R_1R_1$, $R_1^u r$ or $R_0^u r$ cells in low ionic strength
15  saline (LISS).  The anti-D activity of the monoclonals
against a panel of D variant red cells was also
assessed using saline, albumin, papain and IAG
(in LISS) tests.  In a separate series of tests,
the reactivity of the monoclonal anti-Ds of A1,
20  A2, A3, B1, B3 and B11 with $D^u$ red cells (15 sets
of $R_1^u r$ cells and 10 sets of $R_2^u r$ cells, each set
being taken from a different individual) was assessed
by IAG (in LISS) using untreated supernatants.
Using the same procedure, culture supernatant of
25  B1 was further compared with culture supernatant
of B2 against 1 set of $R_1^u r$ cells and 3 sets of
$R_0^u r$ cells.  The supernatants were tested on a Technicon
Autogrouper 16C at dilutions ranging between 1:5
to 1:10,000.

30


Tests against cells of "normal" RhD positive
or RhD negative phenotypes by albumin, papain and
indirect antiglobulin techniques revealed that
35  all the IgG1 monoclonal antibodies showed specificity
for the D antigen (see Table VI below).  None of

the monoclonal anti-Ds were reactive by saline. When tested by IAG against "partial" D positive cells, the antibodies agglutinated $D^{IV}$, $D^V$ and $D^{VII}$ red cells, but not $D^{VI}$ or $D^B$ (see Table VII below). As shown in Table

5    VIII, the supernatants of A1, A2, A3, B1, B3 and B11 were all found to show stronger reactivity by IAG tests with 25 $D^u$ cells (i.e. weak D) than polyclonal antiserum (the routine reagent employed by the South Western Regional Transfusion Service in the U.K). Using the

10    same IAG test procedure, culture supernatant of B1 was found to exhibit similar or identical reactivity to culture supernatant of B2 against 4 further sets of $D^u$ cells (see Table IX). All the IgG1 monoclonal antibodies reacted more strongly with $R_1r$ (<u>CDe/cde</u>) or

15    $R_1R_1$ (<u>CDe/CDe</u>) cells than with either $D^u$ or $D^V$ cells, though there were some differences in titres between the supernatants. When tested separately on a Technicon Autogrouper 16C, supernatants of A2, B1 and B11 could be used diluted to 1:1,000 for use as a routine anti-D and

20    at 1:10 dilution to distinguish $D^u$ from D negative cells.

<div align="center">

<u>Table VI</u>
<u>Serology of monoclonal anti-Rh(D) antibodies</u>

</div>

| RBC phenotype | $R_1R_1$ | $R_2R_2$ | $R_0r$ | r'r' | r"r | rr |
|---|---|---|---|---|---|---|
| Saline 37°: | 0 | 0 | 0 | 0 | 0 | 0 |
| Albumin 37°C: | 4-5 | 4-5 | 4-5 | 0 | 0 | 0 |
| Papain 37°C: | 5 | 5 | 5 | 0 | 0 | 0 |
| IAG: | 5 | 5 | 5 | 0 | 0 | 0 |

(Grade: 0 to 5)

## Table VII

Reaction of monoclonal anti-Rh(D) antibodies with "partial" D positive RBCs by IAG

| Monoclonal antibody: | A1 | A2 | A3 | B1 | B2 | B3 | B11 |
|---|---|---|---|---|---|---|---|
| $D^{IV}$: (n=1) | 5 | 5 | 5 | 5 | 4 | 3 | 5 |
| $D^{V}$: (n=3) | 5 | 5 | 5 | 5 | 5 | 5 | 5 |
| $D^{VII}$: (n=3) | 5 | 5 | 5 | 5 | 5 | 5 | 5 |
| $D^{VI}$: (n=3) | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| $D^{B}$: (n=5) | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

(Grade: 0 to 5)

## Table VIII

Reactivity of monoclonal and polyclonal anti-Ds with $D^{u}$ RBCs by IAG

(15 $R_1^{u}r$ and 10 $R_2^{u}r$)

| Monoclonal antibody: | A1 | A2 | A3 | B1 | B3 | B11 | Poly-clonal anti-Rh(D) Serum |
|---|---|---|---|---|---|---|---|
| Number with | | | | | | | |
| Grade 6: | 16 | 15 | 11 | 6 | 1 | 5 | 0 |
| Grade 5: | 8 | 9 | 12 | 16 | 7 | 17 | 4 |
| Grade 4: | 1 | 1 | 2 | 3 | 9 | 3 | 12 |
| Grade 3: | 0 | 0 | 0 | 0 | 4 | 0 | 7 |
| Grade 2: | 0 | 0 | 0 | 0 | 4 | 0 | 1 |
| Grade 1: | 0 | 0 | 0 | 0 | 0 | 0 | 1 |

Table X

| Monoclonal antibody: | A1 | A2 | A3 | B1 | B2 | B3 | B11 | B12 | B13 | Anti-Rh(D) serum |
|---|---|---|---|---|---|---|---|---|---|---|
| **% specific lysis:** | | | | | | | | | | |
| Effector KF | -2 | -1 | -2 | 91 | * | 99 | 83 | * | * | 95 |
| Effector KL | * | 13 | * | * | 78 | * | * | * | * | * |
| Effector BW | 5 | 4 | 3 | 56 | * | 73 | 70 | * | * | 92 |
| Effector BK | 4 | 15 | * | 91 | * | * | 81 | 71 | 75 | * |

* not determined

    (iv) U937 Monocyte rosetting and phagocytosis assay
        100µl packed $OR_1R_2$ RBCs were sensitised with
        500ul anti-Rh(D) (previously adjusted to 1 ug/ml)
        at 37° for 60 minutes, washed and resuspended at
        1 x $10^8$ cells/ml in RPMI. U937 cells were taken
    5   in the log phase of growth and cultured for two
        days either in the presence or absence of interferon-ɣ
        (Amersham) at 50 U/ml. 45 x $10^6$ RBCs were then
        added to a pellet of 1.5 x $10^6$ U937 cells and mixed,
        giving a ratio of 30:1. For the rosetting assay,
    10  the cells were incubated at room temperature for
        5 minutes, spun at 600 rpm for 3 minutes and examined
        in a haemocytometer after a further 5-20 minutes.
        Phagocytosis was assessed immediately after incubating
        the cells at 37° for 3 hours. Results were expressed
    15  as the percentage of monocytes with one or more
        adherent or phagocytosed RBCs.
            A comparision of the results obtained wth
        culture supernatants of clones A1, A2, A3, B1,
        B3 and B11 and a conventional polyclonal anti-Rh(D)
    20  serum is given in Figures 1 and 2.

(v)   Macrophage binding assay

RBCs (R$_2$r) (1 volume) were sensitised with
monoclonal anti-Rh(D) (2 volumes of untreated culture
supernatant) and incubated with monocyte-derived
5   cultured macrophages.   The macrophages were stimulated
with 500 U/ml of recombinant immune interferon
(Biogen, Geneva) during the 48 hours prior to their
use in the assay.   The binding of RBCs to macrophages
was assesed microscopically and expressed as the
10   macrophage binding index (= number of red cells
attached to or ingested by 100 macrophages).

Table XI below shows the results obtained
with culture supernatants of clones A1, A2, A3,
B1, B3 and B11.   The ability of these supernatants
15   to bring about RBC-macrophage interaction was very
poor compared to that of the polyclonal anti-Rh(D)
serum (anti-Rh(D) - 43IU/ml), which served as a
positive control.

### Table XI

| Source of anti-Rh(D) | A1 | A2 | A3 | B1 | B3 | B11 | polyclonal anti-Rh(D) serum. |
|---|---|---|---|---|---|---|---|
| Macrophage binding index | 20 | 28 | 24 | 5 | 20 | 17 | 416-500 |

EXAMPLE 2
Solution for Rh(D)-Phenotyping of RBCs

In general, it is preferred for the above
purpose to use a blend of an anti-Rh(D) monoclonal
antibody according to the invention (e.g. B11)

with a further IgG monoclonal antibody having anti- $D^{VI}$
activity, for example the monoclonal antibody of the
cell line B7 (ECACC 87091603) of our International
patent application published under No. W089/02443 on 23
5    March 1989.


Solution for Manual Use


      The final blend is 1:1:1 B11:B7: diluent.
10

Diluent   100 ml 30% Bovine Serum Alubumin
          2.42g $KH_2PO_4$
          2.77g $Na_2HPO_4.2H_2O$
          4.50g NaCl
15        0.2 ml Tween 20 (Trade Mark)
          1.00g $NaN_3$
        To 1.0 litre with distilled water: pH 6.8


This blend can be used in all manual tests for D and $D^u$
20   typing, e.g. microtitre, microplate, IAG.


A blend of 1:1:2 B11:B7: diluent may also be used.


Blend for Machine Use
25

This blend may be used, for example, in a Technicon
autogrouper 16C


Pre-blend reagents B11:B7 1:1
30

For D-Phenotyping (D-positive v. D-negative) the
solution comprises 1:1000 blend: diluent, and for $D^u$
determinations ($D^u$ v. D-negative) the solution
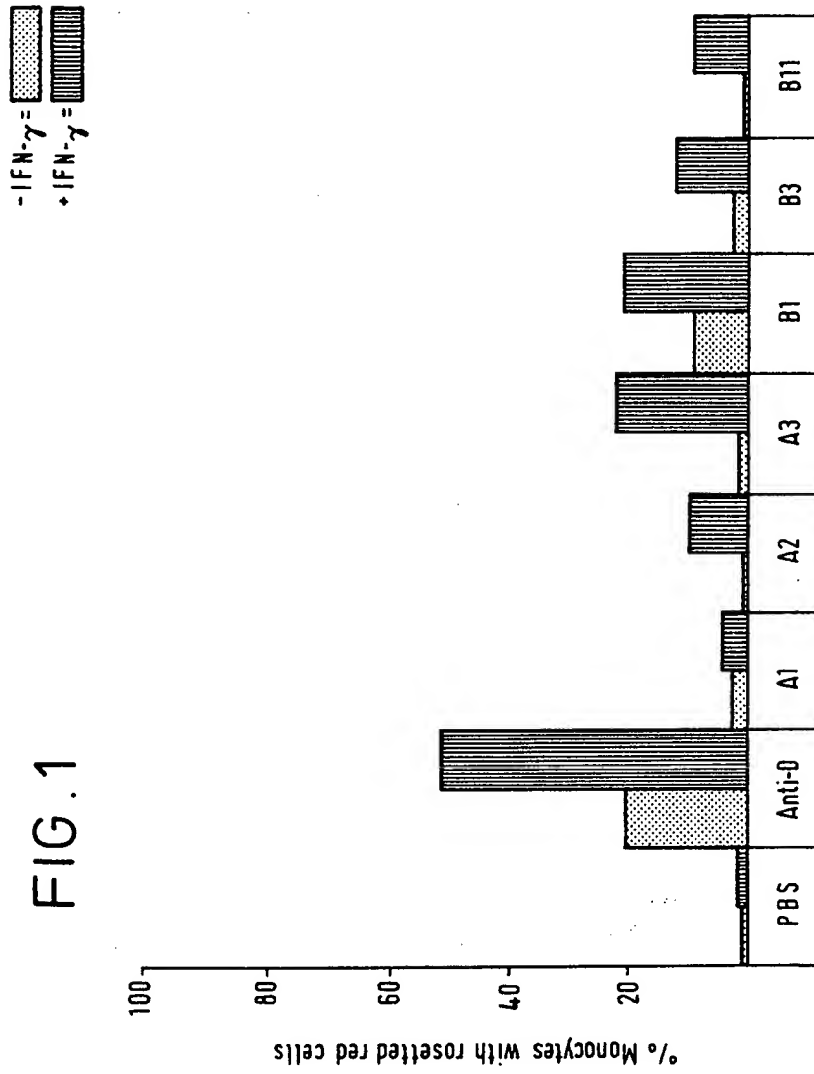comprises 1:5 blend:diluent.
35

Diluent   2% Bovine Serum Albumin in 1.3% physiological
saline containing 13.5% methylcellulose.


5        The monoclonal antibody B7 may be similarly blended
with other antibodies of the present invention, e.g. the
monoclonal antibodies of the deposited cell lines A3
(ECACC 87091605) and B2 (ECACC 87091604) to provide an
anti-Rh(D) reagent with broad specificity for the
10   various D-variant antigens, including $D^{VI}$, and
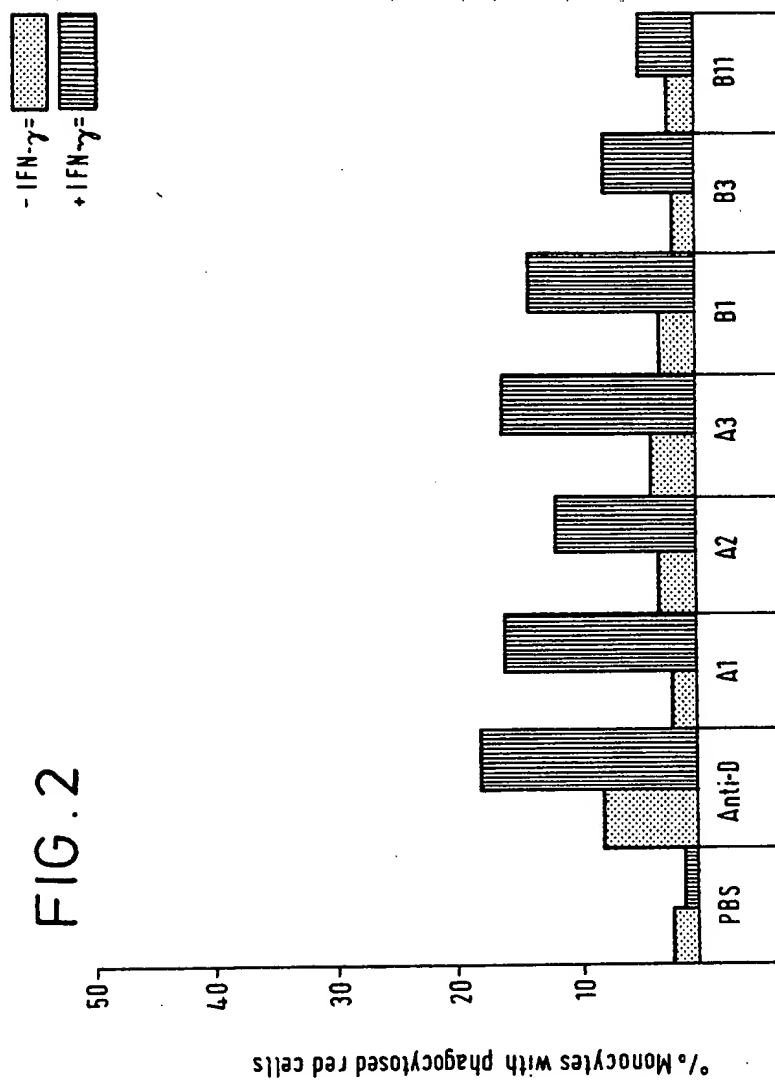exhibiting activity against $D^{u}$ cells by an IAG test.

# FIG.1



*% Monocytes with rosetted red cells*

Legend: -IFN-γ=, +IFN-γ=

X-axis categories: PBS, Anti-0, A1, A2, A3, B1, B3, B11

Y-axis: 20, 40, 60, 80, 100

# FIG.2



legend: -IFN-γ=  +IFN-γ=

Y-axis: % Monocytes with phagocytosed red cells

Scale: 50, 40, 30, 20, 10

X-axis categories: PBS, Anti-D, A1, A2, A3, B1, B3, B11